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RADIOFREQUENCY FIELD EXPOSURE OF CULTURED LYMPHOCYTES FROM 'MAC--ETC(U)
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RADIOFREQUENCY FIELD EXPOSURE OF CULTURED LYMPHOCYTES FROM MACACA MULATTA

INTRODUCTION

For approximately 20 years now, scientists have been exploring the possibility of direct electromagnetic (EM)-field effects on cells in general (e.g., 8) and lymphocytes in particular, both in vivo (5) and in vitro (1, 7). The lymphocyte case is particularly interesting because of its involvement in the immune response of organisms.

Prince et al. (5) reported that exposure to intense radio-frequency fields (RFF) increased the mitotic potential of certain populations of circulating primate lymphocytes. These findings were accompanied by substantial tissue heating, i.e., $+2.4^{\circ}\text{C}$, as well as 4.6°C rectal and surface temperature increase, following 26.6-MHz RFF exposure at 1.32 W/cm^2 incident power for 30 minutes. Czerski (1) had reported earlier relatively athermal EM-field-induced lymphoblastoid transformations in human lymphocyte populations cultured and exposed in vitro. However, even Czerski's cultures underwent a $+1.0^{\circ}\text{C}$ change during the course of exposure. In addition, the field patterns in the culture container employed by Czerski could well have promoted local "hot spots" despite the relatively small temperature changes reported.

In an attempt to extend the Prince et al. (5) work on primate lymphocytes to a thermally controlled in vitro situation, we initiated a series of studies employing a temperature-controlled RF-exposure culture cup (2) that also provides for well-defined RF-field conditions. Thus, in our early studies we attempted to determine if cultured primate lymphocytes exposed to intense ($E=500\text{ V/m}$; $H=4.4\text{ A/m}$; $\text{SAR}=400\text{ W/kg}$) 30-MHz fields would yield significantly higher mitotic figures than sham-exposed control samples of cultured lymphocytes, while the temperature of the cultures under both conditions was held at $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$.

In the first series of studies we examined extensively the effects of 30-MHz fields on primate lymphocyte time-dependent mitotic figures and blast cell counts. Although a great deal of data were gathered, the results were equivocal in delineating any possible role of RF radiation in vitro on lymphocyte mitotic processes (3). However, the rate of success in bringing to harvest RF-exposed lymphocytes was substantially less (68.6%) than the success rate for bringing to harvest sham-exposed primate lymphocytes (92%). With this in mind, our terminal study of the series compared pre- and postradiation lymphocyte counts as determined by hemocytometer. The determinations were made on pair-matched samples (i.e., taken from the same primate at the same time), and the results of these determinations suggested that exposure for 20 minutes in the temperature-controlled culture cup resulted in a statistically reliable difference in the number of viable lymphocytes.

Primate lymphocytes exposed to RF-fields produced lower viable cell counts than pair-matched samples that spent an equivalent amount of time in a temperature-controlled waterbath (both $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$). On the other hand, this difference was not found when 4 pair-matched samples were either sham exposed (0 V/m) or placed in the same temperature-controlled waterbath. Although the waterbath and temperature in the culture cup were controlled at approximately $37.0^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$, the matched samples were not "yoked" as such, i.e., controlled by the same constant temperature circulator "plumbed" in series. The results of these 30-MHz pre- and postexposure comparisons are shown in Table 1 (500 V/m and Table 2 (0 V/m).

TABLE 1. NUMBER OF VIABLE CELLS ($\times 10^6$ /CULTURE) FOLLOWING 20-MINUTE EXPOSURE TO 30-MHz FIELDS AT 500 V/m COMPARED TO NUMBER OF CELLS FROM PAIR-MATCHED SAMPLE MAINTAINED IN TEMPERATURE-CONTROLLED CULTURE CUP

500 V/m	Control
1.27	1.32
1.70	2.00
2.01	2.38
2.52	3.03

Sandler's A = .3235, df = 3, $p < .05$
(ref.6)

While 2 sets of 4 pair-matched samples are minimal data, the statistically reliable outcome was nonetheless consistent with our lower success rate for culturing primate lymphocytes following RF-field exposure, and suggested that additional studies should be conducted to determine if cell death or damage was induced in primate lymphocytes exposed in vitro to intense 30-MHz fields. These latter studies were conducted with the intent of employing more sophisticated cell-counting techniques (e.g., Coulter Counter) than were employed in the earlier studies (hemocytometer counts). In addition, we planned to examine the effects of RF-field exposure on both phytohemagglutinin (PHA)-stimulated as well as nonstimulated lymphocytes. Finally, in addition to the counts obtained from the Coulter Counter we randomly selected a number of culture samples to determine microscopically from vitally stained smears if there was any evidence of dead lymphocytes or cellular debris indicative of damage (e.g., membrane rupture).

TABLE 2. NUMBER OF VIABLE CELLS ($\times 10^6$ /CULTURE) FOLLOWING 20-MINUTE EXPOSURE TO 0 V/m COMPARED TO NUMBER OF CELLS FROM PAIR-MATCHED SAMPLE MAINTAINED IN TEMPERATURE-CONTROLLED CULTURE CUP

0 V/m	Control
3.05	3.00
3.70	3.70
3.55	4.51
0.672	0.755

Sandler's A = .944, df = 3, NS (ref. 6)

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METHODS AND MATERIALS

RF Radiation Effects on Lymphocytes Not Stimulated with PHA

The subjects were 4 adult male rhesus monkeys (*Macaca mulatta*) restrained and tranquilized with ketamine (10 mg/kg, I.M.). Ten-milliliter samples of arterial or venous blood were drawn into a heparinized syringe. Whole blood was then centrifuged at 600 rpm for 20 minutes, and the white cell layer of the suspension was pipetted off and mixed with culture media (Eagles' MEM, 10% fetal calf serum, penicillin-streptomycin, and glutamine added) in a 3:7 ratio; the total volume then being split into two 5-ml samples. From each of the 5-ml culture samples a 0.5-ml aliquot was removed and placed in a capped test tube for the preradiation cell counts using a Coulter Counter (Model F). Following removal of the preradiation sample, 0.5 ml of culture media was added to the remaining 4.5-ml culture volume to restore total volume to the 5-ml capacity of the RF-exposure culture cup. Because of the different cell concentrations in the preradiation and postradiation samples, the former cell counts were multiplied by a factor of 0.9; i.e., the differential cell density of the 2 samples was taken into account.

The 5-ml samples were either radiated or sham radiated in the stainless-steel culture cup to 30-MHz fields at a field strength of 500 V/m for 20 minutes. The temperature of the cell cultures during exposure was maintained at $37^\circ\text{C} \pm 0.5^\circ\text{C}$ by a constant-temperature circulator.

Following exposure, the cultures were returned to culture test tubes and a 0.5-ml sample was removed for the postradiation cell count. The rest of the culture was then incubated at 37°C in a CO₂-enriched environment. Approximately 24 hours later another 0.5-ml sample was taken from the incubated cultures for the final cell count.

RF Radiation Effects on Monkey Lymphocytes Stimulated with PHA

Lymphocyte culture preparation procedures for the PHA phase of the research were very similar to that for the non-PHA-stimulation studies. Briefly, blood was taken from the same 4 male monkeys, and their white blood cells were separated by centrifugation. Lymphocyte cultures were established by adding white cells to culture media except two 5.5-ml culture samples were obtained instead of the 5-ml samples. The concentrations of cells in the pre- and postradiation samples were the same. In addition, 0.2 ml of PHA-L (Difco: diluted 50:1 from dried stock) was added to each 5.5-ml culture approximately 30 minutes before radiation or sham radiation in the culture cup. After the addition of the PHA to the lymphocyte culture preparation, and again immediately following the radiation or sham radiation, and, finally, again at 48 hours after RF exposure, a 0.5-ml sample from each culture was sent to the Department of Laboratory Medicine for a complete count on a Coulter Counter (Model F). In addition to these determinations, the Department of Laboratory Medicine also did vital staining of randomly selected cultures that consistently revealed no dead cells nor cell fragments. Thus, no uptake of the gentian violet nor the trypan blue occurred in the cultures selected for vital staining; based on these minimal data, one is forced to conclude that RF exposure did not induce cell death nor gross morphological change, e.g., membrane herniation in PHA-stimulated lymphocytes. It should be noted that the vital stain studies were carried out by two independent investigators in the Department of Laboratory Medicine with one of the two making "blind" assessments. The two investigators arrived at the same conclusions. The Department of Laboratory Medicine also attempted to perform a number of hemocytometer counts on cultures stimulated with PHA, but discontinued the procedure as a result of severe cell clumping (the same difficulty we encountered when we attempted to do our own counts on PHA-stimulated lymphocytes).

In addition to the pre- and postexposure Coulter counts, a 48-hour determination was made in the PHA-stimulated culture tests because a 48-hour delay extended the culture time to the low end of the time domain employed in our earlier mitotic figure work as well as slide preparation, vital staining, and microscopic examination for cell debris or whole cell death.

RESULTS AND DISCUSSION

After initial failures to achieve consistent precision in cell counting with a hemocytometer, arrangements were made to have our cell counts done on a Coulter F counter in the Hematology section of the Department of Laboratory Medicine in the University Hospital. The pre- and postradiation samples were counted on one day, and the 24-hour incubation samples were counted on the following day. The cell counts are relative numbers which can be compared only to the counts done on the same day, and the counts are quite variable for the following reasons:

1. In the Department of Laboratory Medicine, clinical tests have higher priority than the research tests. Research samples are counted when the clinical work permits. Therefore, the time from when the samples were sent to the laboratory to the time the cells were actually counted varied from minutes to hours. Also, the handling of samples varied daily with different technicians.

2. Before counting, 0.02 ml of our sample was diluted to 10-ml isotonic solution (Isoton II). This dilution process still gives accurate counts for clinical tests because of the high concentration of white blood cells. However, in our relatively low concentration samples, the error in counts was larger.

3. The background counts were checked by running Isoton II through the Coulter Counter. These counts were highly variable and dependent on the setting and maintenance of the counter. While the background counts usually appear reasonable for clinical assessment, they at times seem high for our relatively low cell counts. On many occasions, the background counts were not even reported to us.

Results of RF Exposure on Unstimulated Lymphocytes

Despite the procedural limitations enumerated above, we were still able to complete a number of "clean" runs. The results are presented in Table 3, which shows 8 pair-matched samples.

The counts presented in Table 3 were normalized to the preradiation sham counts. The "blank" figures in the table represent the counts obtained by running Isoton II plus culture media (without cells) through the counter. The units are expressed in terms of counts per cubic millimeter of solution. Comparison of the reported background counts and our "blank" figures showed that the culture media alone did not change the background counts significantly.

TABLE 3. RF-RADIATION EFFECTS ON MONKEY LYMPHOCYTES (SINGLE RUNS)

		Normalized cell counts		
		Before exposure	After exposure	24 hrs after incubation
Pair 1:	Sham	1	1	1.07
	Exposed	1.14	1.45	1.22
Pair 2:	Sham	1	0.49	1.11
	Exposed	1.07	0.58	1.32
	Blank (count/mm ³)	0.1		0.17
Pair 3:	Sham	1	0.86	0.95
	Exposed	0.93	0.87	1.02
	Blank (count/mm ³)	0.2	0.4	0.9
Pair 4:	Sham	1	0.95	0.56
	Exposed	0.82	0.77	0.59
Pair 5:	Sham	1	0.97	0.60
	Exposed	1	1.11	0.32
	Blank (count/mm ³)	1.0	1.0	1.3
Pair 6:	Sham	1	0.99	0.75
	Exposed	1.19	1.14	0.85
	Blank (count/mm ³)	1.37	0.87	0.4
Pair 7:	Sham	1	1.09	1.05
	Exposed	1.09	1.13	0.84
Pair 8:	Sham	1	1.08	1.24
	Exposed	1	1.24	1.57
	Blank (count/mm ³)	0.3	0.1	0.1

The 8 pair-matched runs presented in Table 3 are briefly summarized in Table 4, which at first glance suggests that there is little, if any, effect on the viability of primate lymphocytes exposed to intense 30-MHz fields whether the determination is made immediately after exposure or 24 hours later relative to the sham-exposed, pair-matched controls. However, statistical evaluation of the difference scores (Table 3) between matched pairs reveals that the postradiation normalized counts are significantly different when counted immediately after exposure ($A=.247$, $df=7$, $p<.05$) but not when counted 24 hours after incubation ($A=.43$, $df=7$, NS). Nevertheless, taking all 16 paired post-exposure values into consideration reveals that 13 of the 16 pairs yield lymphocyte counts which are higher in the exposed samples than in the sham-exposed samples. This finding is statistically reliable ($p=0.011$, by Sign test).

These findings, summarized in Tables 3 and 4, are at variance with our report that lymphocyte viability is reduced (see Tables 1 and 2) consequent to 20-minute exposure to 30-MHz fields. Indeed, we appear to have more viable cells. We are at a loss to explain this finding, one diametrically opposed to our earlier suggestion of lower viability when culturing to obtain white-cell mitotic figures (3,4). It should be pointed out that the earlier mitotic figure work employed the mitogen PHA, while the results reported in Tables 3 and 4 here represent the outcome of nonstimulated cells undergoing RF exposure. Nevertheless, our earlier study employing a pair-matched design to examine RF exposure effects on lymphocyte viability also failed to employ a mitogen and this also resulted in a statistically reliable loss in white cells. One possible explanation for the discrepancy is that the original finding (Table 1) was the result of a false positive, and that the earlier reduction in culture viability rate following RF exposure was related to the presence of the mitogen PHA. Thus, it is conceivable that PHA-stimulated primate lymphocytes might somehow be more susceptible to possible adverse effects of exposure to RF radiation. This possibility has been evaluated by the methods outlined above and the results summarized in the following section.

TABLE 4. SUMMARY OF RF-RADIATION EFFECTS ON MONKEY LYMPHOCYTES

	<u>Normalized cell counts</u>		
	<u>Before exposure</u>	<u>After exposure</u>	<u>24 hrs after incubation</u>
Sham	1	0.95 ± 0.21	0.92 ± 0.25
Exposed	1.12 ± 0.33	1.04 ± 0.28	0.97 ± 0.40

Results of RF Exposure on PHA-Stimulated Lymphocytes

The Coulter Counter PHA-stimulated lymphocyte counts were preradiation, postradiation, and 48-hour determinations as summarized in Table 5 and expressed as difference scores between a given exposed culture and its pair-matched sham-exposed control.

Statistical evaluation of the data in Table 5 reveals nonsignificant difference between matched pairs counted as preradiation samples ($A=.922$, $df=12$, NS) or immediate postradiation samples ($A=.777$, $df=12$, NS). On the other hand, the 48-hour postradiation determinations were significantly different ($A=.226$, $df=10$, $p<.05$) in the same direction as the nonstimulated findings of Tables 3 and 4. Finally, the average of the difference scores taken over time suggests that a clear trend in elevated counts develops over time, postradiation.

In summary, the Coulter Counter determinations of primate lymphocyte viability are consistent, despite the run-to-run variability, in suggesting small but significant elevations in viable primate lymphocytes following exposure to 30-MHz fields. The findings seem to be independent of the addition of PHA (if one ignores time dependence). Finally, the Coulter Counter determinations, while at odds with our earlier hemocytometer evaluations (Tables 1,2), were supported by the vital staining and microscopy done on randomly selected samples. These latter evaluations provided no evidence of cell damage, death, or other anomaly induced by exposure to 30-MHz RF radiation. Thus, our earlier reports (3, 4) of lymphocyte damage consequent to 20-minute exposure to intense ($SAR=400$ W/kg), temperature-controlled, 30-MHz fields are simply not supported by the data of either study reported here.

TABLE 5. COULTER COUNTER DIFFERENCE SCORES (RADIATED LESS SHAM
RADIATED) FOR PAIR-MATCHED, PHA-STIMULATED PRIMATE
LYMPHOCYTES

Culture sample	Preradiation	Immediate postradiation	48-hour postradiation
1	(-) 0.076	(-) 0.193	NA*
2	(+) 0.032	(-) 0.350	(+) 0.350
3	(-) 0.054	(-) 0.131	(-) 0.162
4	(-) 0.053	(-) 0.084	NA
5	(+) 0.052	(+) 0.010	(+) 0.246
6	(+) 0.019	(+) 0.461	(+) 0.033
7	(-) 0.106	(-) 0.151	(+) 0.410
8	(-) 0.012	(+) 0.339	(-) 0.020
9	NA	(-) 0.220	(-) 0.010
10	(-) 0.150	(+) 0.050	(+) 0.139
11	(-) 0.410	NA	NA
12	(+) 0.090	(+) 0.320	(+) 0.350
13	(+) 0.120	(+) 0.500	(+) 0.010
14	(+) 0.130	(+) 0.238	(+) 0.110
N =	13	13	11
Mean difference	(-) 0.032	(+) 0.061	(+) 0.132

*NA = not available

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